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To: Dr. J. L. Charles **Date:** February 26, 1988

From: C. Ellis and M. Penn

Subject: Operational Plans for the Lowered Biological Activity Program

OBJECTIVE: To decrease the biological activity of cigarette smoke condensate (CSC) by 90% as determined by in vitro assays by a target date of 1992.

BACKGROUND AND STATUS: Based on information in the literature, biological activity can be divided into several stages: initiation, promotion, and conversion. In addition, various biochemical factors may modulate the activity of a substance. The strategies used in the Lowered Biological Activity Program have been to: determine which stage(s) may be important the activity of CSC; develop in vitro bioassays to quantitatively measure CSC activity; evaluate cigarette models in the assays to obtain background information; develop and test models which are reduced in activity; and optimize the subjectives of a low activity model.

After many years of investigating assays for their ability to detect initiation activity, the Salmonella/microsome (S/M) assay was determined to provide the best information for our purposes. In addition, the S/M assay is the most widely used and accepted in vitro bioassay in the scientific community. The S/M assay is currently being used in the Crossed Solubles/Base Web Study to develop model cigarettes with reduced activity. Feedstock, base web, and all possible RL combinations of the solubles and base webs were made from burley, bright and oriental tobacco. Extensive analytical data have been obtained in these models and, based on this information, modifications have been made to the solubles by a variety of methods. The effect of these modifications on activity is evaluated by spraying the solubles onto the base web, fabricating cigarettes, and testing the CSC in the S/M assay. Significant reductions in activity have been obtained with various modifications to the CEL and an active research program is planned in this area.

Based on the lower S/M activity models that have been developed, work will need to be done to improve the subjectives of these cigarettes. Studies involving the optimization of low activity models have concentrated on the reduction of carbonyls in smoke. This work has been successful and future studies will focus on the reduction of NO_x in smoke.

The Glutathione Depletion Assay (GDA) is a biochemical endpoint that is utilized to evaluate the effect of CSC on glutathione depletion. Glutathione (GSH) is a low molecular weight thiol that is important to normal cellular functions and biological defense mechanisms. Depletion of GSH has been demonstrated to produce an increase in the activity of a positive control

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compound in the S/M assay. Plans in this area include the evaluation of a variety of models and the isolation and identification of substances in CSC that are responsible for GSH depletion.

The development of a promotion assay for CSC continues to be a key goal of the Lowered Biological Activity Program. The central focus of the potential promotion assays under development is the enzyme protein kinase C (PKC) which can modulate cell physiology and biochemistry via the phosphorylation of cellular proteins. The two binding assays that are currently being investigated are the epidermal growth factor (EGF) binding assay and the phorbol 12, 13 - dibutyrate (PDBu) binding assay. The results obtained with the EGF assay indicate that CSC obtained from several cigarette models can inhibit EGF binding to its receptor. Investigations regarding the assay's ability to differentiate among selected CSCs are in progress. Optimization of assay parameters and kinetic studies for the PDBu binding assay have been completed. Experiments are now being conducted to obtain a positive response with IT 2R1 CSC. Two additional assays are being developed to measure the effect of CSC on protein kinase C in cell fractions and intact cells.

Because some aspects of biological activity are poorly understood and are the subject of intense investigation in the scientific community, additional leads may become available to us at any time. New information could drastically affect our plan. We intend to monitor the literature very closely and make modifications to the program when the evidence warrants a change.

STRATEGIES:

1. BIOASSAY DEVELOPMENT: develop suitable in vitro bioassays which can clearly differentiate among a series of model cigarettes.

2. MODEL EVALUATION: determine the activity of smoke condensate from various types of cigarette models in order to obtain background information from which to design modifications.

3. MODEL DEVELOPMENT: perform modifications to various cigarette parameters to reduce activity and formulate new models.

4. MODEL OPTIMIZATION: improve the subjectives of a low activity model.

TACTICS AND TIMETABLE:

OPERATIONAL PLANS FOR 1988

I. BIOASSAY DEVELOPMENT

A. Epidermal Growth Factor Binding Assay

First Quarter 1988

1. Determine the effect of cell density on EGF binding. (February 1)

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2. Begin to evaluate the effects of CSC fractions on EGF binding. (March 1)

Second Quarter 1988
1. Examine the time course of the CSC effect. (May 1)
2. Complete the evaluation of CSC fractions. (June 1)

Third Quarter 1988
1. Test CSCs from other model cigarettes. (July 1)
2. Using the EGF assay, investigate the activity of other classes of promoters. (September 1)

Fourth Quarter 1988
1. Begin to examine the pathway through which CSC inhibits EGF binding to aid in the development of a biochemical assay. (September 1)

Decision Point on EGF Assay: December 1988

B. Inhibition of Phorbol 12,13 -Dibutyrate (PDBu) Binding

First Quarter 1988
1. Obtain a response with 2R1 CSC. (March 1)

Second Quarter 1988
1. Optimize the response and determine the quantitative properties of the assay. (May 1)
2. Examine the effects of two model CSCs and catechol. (June 1)

Third Quarter 1988
1. Examine the effect of CSC on the affinity and number of PKC receptors. (July 1)

Decision Point on PDBu Assay: September 1988

C. Protein Kinase C (PKC) Activity Assay

First Quarter 1988
1. Examine the effect of CSC on the basal levels of the enzyme (time course and dose response experiments). (March 31)

Second Quarter 1988
1. Examine the effect of CSC on stimulated levels of the enzyme. (June 30)

Decision point on the PKC Activity Assay: July 1988

D. Protein Kinase C Activity in Intact Cells

1. Optimize assay conditions to obtain a positive response with TPA. (March 30)

Second Quarter 1988

1. Develop methods for quantitation (obtain appropriate software). (Begin April 1)
2. Determine the effects of pre or post labeling with a promoter. (May 30)
3. Examine the labeling patterns of 3T3 cells with the PhastSystem using a variety of cell conditions (logarithmic, confluent, and serum-deprived) (July 1)

Third Quarter 1988

1. Complete a preliminary investigation of the effects of CSC. (October 1)

Fourth Quarter 1988

1. Perform additional experiments as needed on CSCs or fractions. (December 31)

Decision Point on the PKC Activity Assay in Intact Cells: December 1988

II. MODEL EVALUATION

A. Salmonella/microsome (S/M) Assay

First Quarter 1988

1. Evaluate the effects of filters (charcoal, THAM, basic alumina) on the S/M activity of washed shredded bright stem and BCR blend cigarettes. (March 1)

B. Glutathione Depletion Assay (GDA)

First Quarter 1988

1. Begin a literature search on the isolation and identification of GSH adducts. (February 1)
2. Evaluate the gas phase and whole smoke of BCR blend cigarettes with whistle through, THAM on charcoal, and basic alumina filters. (March 31)

Second Quarter 1988

1. Determine of effects of GSH depletion by CSC on activity measured in the S/M assay. (June 30)

Third Quarter 1988

1. Evaluate the activity of HCN in the GDA. (August 1)
2. Begin isolation and identification of GSH-CSC adducts. (August 1)

III. MODEL DEVELOPMENT

A. Crossed Solubles/ Base Web Study

First Quarter 1988

1. Compile and issue a report containing analytical data. (March 31)

2. Begin an examination of the effect of ultrafiltration and size exclusion techniques. (January 1)
3. Begin an examination of selective ion exchange resins. (January 1)
4. Begin investigation of the effects of monovalent and divalent cations. (February 1)

Second Quarter 1988
1. Complete survey study of ultrafiltration and size exclusion techniques. (May 1)
2. Complete survey study of ion exchange resins. (June 1)

Third Quarter 1988
1. Begin investigation of effects of nitrogenous compounds on activity (amino acids, proteins, nitrate). (July 1)
2. Complete investigation of the effect of monovalent and divalent cations. (September 1)

Fourth Quarter 1988
1. Investigate the effect of sugar-chlorogenic acid or sugar-caffeic acid complexes on activity. (December 1)
2. Begin to investigate the effects of combining various modifications. (December 1)
3. Determine the effect of CEL insolubles on activity. (December 31)

IV. MODEL OPTIMIZATION

A. Carbonyl Reduction

First Quarter 1988
1. Evaluate the selective reduction of both formaldehyde and acrolein. (February 15)

B. NO_x Reduction

First Quarter 1988
1. Complete a literature search. (February 15)
2. Obtain and evaluate necessary papers. (March 15)
3. Investigate and establish analytical procedures suitable for NO_x determination in MS smoke. (March 15)

Second Quarter 1988
3. Design prototype filters for the reaction/adsorption of NO_x. (April 15)

EXTENDED PLANS:

BIOASSAY DEVELOPMENT:

1989: Begin to investigate other biochemical endpoints and techniques (mouse epidermal cell cultures, free radical mechanisms, intracellular calcium). Investigate other endpoints as evidence warrants. Begin to develop flow cytometry techniques.

1990: Utilize Flow Cytometry to increase our sensitivity, efficiency, and capabilities with regard to in vitro bioassays.
1991: Develop an assay for promotion activity. Investigate the activity of CSC in assays measuring activity in other stages of Biological Activity.

MODEL EVALUATION:

1989-1992: Continue to evaluate models as needed.

MODEL DEVELOPMENT:

1989-1990: Combine all effective modifications to obtain a low activity model for the S/M assay. Begin extensive testing. Begin flavor work.
1991: Begin the development of low promotion activity models.

MODEL OPTIMIZATION:

1989-1990: Optimize the subjectives of a low S/M activity model.
1991: Begin work on optimizing the subjectives of a low promotion activity model.

RESOURCE ALLOCATIONS FOR 1988:

Personnel Allocations:

BIOASSAY DEVELOPMENT:
EGF Assay- G. Patskan(100%), D. Stagg(100%), B. Davies(10%), M. Penn (10%)
PDBu Assay- T. Ferguson(100%), B. Davies (20%), M. Penn (10%)
Protein Kinase C Activity- B. Vaughan(60%), B. Davies(20%), M. Penn(10%)
Protein Kinase C Activity in Intact Cells- G. Nixon(100%), B. Davies(15%), M. Penn(10%)
Collen Deubler assists part-time in all of the above(25%).

MODEL EVALUATON:
S/M Assay- L. Thompson(100%), S. Coleman(75%), C. Deubler(50%), M. Penn(10%)
GDA- B. McCoy(100%), B. Vaughan(40%)
Smoke and Sample Preparation: R. Hellams(40%), N. McGee(45%), R. Kinser (5%)

MODEL DEVELOPMENT:
Crossed Solubles/Base Web Study: D. Williams (25%), S. Coleman (25%)
Smoke and Sample Preparation: R. Hellams(40%), N. McGee(45%), R. Kinser (5%)

MODEL OPTIMIZATION:
Carbonyls and NO_x: B. Levins(65%), R. Kinser (10%)

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Number of People:

There are immediate needs in the following areas:

1. A replacement for D. Stagg (T4) who is on special assignment. It is imperative that this person would require little training and have experience with sterile technique, mammalian cell culture, and handling radioactive materials. Due to the nature of our work, the use of a temporary employee in this area is not feasible. Because of the anticipated time frame involved, utilizing a technician who will require a significant amount of training would not provide us with an adequate return on our training investment. The feasibility of obtaining a qualified person from another area and replacing that person with a temp has been explored. Neither Bob McCuen nor Dick Cox had a T4-level individual with the appropriate qualifications. Based on this information and the uncertainty surrounding the duration of Ms. Stagg's assignment, I recommend that a full-time technician be hired to assist us in this area. Failure to do so will have a negative impact on the schedules outlined in this document.

2. A Chemist (replacement for David Williams who was on loan to us from Chemical Research) with some biology experience (Scientist or above) who can perform the following: crossed soluble/base web modifications and separations; coordinate model optimization (flavors), assist with the isolation and identification of GSH adducts, provide general assistance and chemical expertise to the Lowered Biological Activity Program.

3. A Chemist (Associate B level) who can assist in model development: the preparation of model cigarettes, spraying filler with modified CEL or other substances of interest, preparing cigarettes, preparing LTF formulations, and performing chemical analyses on filler and smoke. The individual would work with R. Hellams and assist in research programs on the effect of filtration and smoking parameters on biological activity.

Anticipated need in 1989 is:

1. An individual with experience in flow cytometry. This relatively new technique could greatly enhance the sensitivity, efficiency, and capability of our bioassays and would impact Bioassay Development and other related areas of research. It is estimated that we could utilize these skills in 1989. A thorough review of the capabilities of this technique will be made in 1988 and an extensive justification will be written.

Skills of People:

See above.

Special Equipment and Facilities:

A Flow Cytometer (about $500K) could be utilized in 1989. This piece of equipment would have a significant impact on the sensitivity, efficiency, and capabilities of much of the biology/biochemistry related work. A thorough justification will be developed in 1988.

Outside Expertise:

Occasional antibody work may need to be performed.

Impact on Other Areas of PM:

1988:
Analytical Research: 200 man hours/year
Cigarette Testing: 30 man hours/year
Pilot Plant: 2 weeks every two years
Semiworks: approximately 1/2 day/year

Extended:
Chemical Research: flavor development, beginning in 1989
NMR/MS(probably LC or FAB): for the isolation and identification of glutathione-CSC adducts, beginning in 1989
Flavor Development: assistance beginning in 1990

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